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[51] Fluorescence and Optical Characteristics of Reduced Flavins and Flavoproteins

By SANDRO GHISLA

Flavins and flavoproteins in their *oxidized* state (Fl_{ox}) are, as the name indicates, typically yellow, with the absorption spectrum in the visible and near-ultraviolet (UV) range being characterized by two well-resolved bands centered around 450 and 370 nm. This chromophore, free in solution, exhibits a strong fluorescence that is maximal around 520 nm; when the chromophore is bound to flavoproteins, however, its emission can be fully quenched. These favorable properties have promoted many detailed spectroscopic investigations dealing with theoretical aspects, with model compounds, and with flavoenzymes[1-5]; see also this volume [50].

[1] Several articles in T. P. Singer, ed., "Flavins and Flavoproteins." Elsevier, Amsterdam, 1976.
[2] P. S. Song, *in* "Flavins and Flavoproteins," (H. Kamin, ed.), p. 37. Univ. Park Press, Baltimore, Maryland, 1971.
[3] M. Sun, T. A. Moore, and P. S. Song, *J. Am. Chem. Soc.* **94**, 1930 (1972).
[4] J. L. Fox, S. P. Laberge, K. Nishimoto, and L. S. Forster, *Biochim. Biophys. Acta* **109**, 626 (1965).
[5] F. Müller, S. G. Mayhew, and V. Massey, *Biochemistry* **12**, 4654 (1973), and articles cited therein.

In contrast, *reduced* flavins and flavoenzymes (Fl_{red}) have a rather atypical and variable absorption above 300 nm and have long been considered to be nonfluorescent.[6] This, and the technical problems deriving from the sensitivity of reduced flavins toward oxygen, probably have discouraged similar detailed investigations. An understanding of reduced flavins, however, is even more relevant than an understanding of oxidized flavins. In fact, in addition to being the counterpart of Fl_{ox} in redox reactions, reduced flavins activate molecular oxygen in flavin-dependent oxidases, in monooxygenases, and in bacterial luciferase.[7,8]

Methods of Preparation of Reduced Flavins and Flavoproteins

Basically any reducing agent of suitable potential will reduce free oxidized flavins ($E_0' \simeq 200$ mV) to the 1,5-dihydro level. In the case of some flavoproteins, kinetic barriers, or the low potential for reduction of the semiquinone to the dihydro form, can render full reduction very difficult to attain.[9] The choice of the reducing agents will depend on the scope of the investigation planned; for this reason, the most frequently employed methods will be briefly discussed below. When the reduction is carried out to obtain reduce flavins for spectroscopic measurements, Tunberg-type cuvettes are best employed. An apparatus suitable for such experiments has been described by Foust *et al.*[10] This set-up can be modified most conveniently by use of an air-tight Hamilton syringe glued into an appropriate ground-glass joint, instead of the original[10] titration glass burette.

Reduction Procedures

Dithionite. This agent can be either a one- or a two-electron reducing agent.[11,12] Its potential of -0.66 mV[9] is sufficiently low for the reduction of most flavoproteins; some of those which we have reduced by this method are listed in the table. Dithionite itself has a rather strong absorption at 315 nm ($\epsilon \simeq 5500$–8000)[8]; therefore, the addition of excesses

[6] S. Ghisla, V. Massey, J. M. Lhoste, and S. G. Mayhew, *Biochemistry* **13**, 589 (1974).
[7] V. Massey and P. Hemmerich, *in* "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 12, p. 191, Academic Press, New York, 1975.
[8] H. Bright and D. J. T. Porter, *in* "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 12, p. 421, Academic Press, New York, 1975.
[9] S. G. Mayhew, *Eur. J. Biochem.* (in press).
[10] G. P. Foust, B. D. Burleigh, S. G. Mayhew, C. H. Williams, and V. Massey, *Anal. Biochem.* **27**, 530 (1969).
[11] S. G. Mayhew and V. Massey, *Biochim. Biophys. Acta* **315**, 181 (1973).
[12] D. O. Lambeth and G. Palmer, *J. Biol. Chem.* **248**, 6095 (1973).

Enzyme	Coenzyme	Oxidized state: Absorption λ_{max} (nm)	Oxidized state: Absorption ϵ (10^3 M^{-1} cm^{-1})	Oxidized state: Fluorescence λ_{max} (nm)	Oxidized state: Fluorescence Rel. inten. (% of FMN)	Reduced state: Absorption λ_{max} (nm)	Reduced state: Absorption ϵ (10^3 M^{-1} cm^{-1})	Reduced state: Fluorescence λ_{max} (nm)	Reduced state: Fluorescence Rel. inten. (% of FMN)	Method of formation	Reference[b]
Lactate oxidase	FMN	460 373	11.3	—	—	360	4.8	507	7.0	$NaBH_4$, substrate $Na_2S_2O_4$	6
N(5)-CH_2-Coo^- adduct	1,5-FMN_{red} (modified)					365	5.0	476	34	red. enzyme + $BrCH_2COO^-$	6
N(5)-CHOH-COO^- adduct	1,5-FMN_{red} (modified)					365	5.0	465		CH_2OH-COO^- (as substrate)	31
C(4a)-N(5) adduct	4a,5-FMN_{red} (modified)					368 318	5.0 9.0	502	30	H-C≡CHOH-COO^- (as substrate)	30
C(4a)-N(5)-propano	4a,5-FMN_{red} (modified)					375 315s	5.0 9.0	530		Chemical modif. of coenzyme	30
C(4a)-OH,N(5)-C_2H_5	4a,5-FMN_{red} (modified)					375 320		530		Chemical modif. of coenzyme	25
Glucose oxidase	FAD	452 383	14.1 13.3	—	—	355	6.2	—	—	$NaBH_4$	6
D-Amino acid oxidase	FAD	455	11.3	530	1.5	415s	2.9			Substrate, $Na_2S_2O_4$	6
		380	9.7			355	4.1			EDTA-hν	
C(4a)-CH_2-C_6H_5, adduct	4a,5-FAD_{red} (modified)					365 320s	5.8 7.4	520	3.9	$C_6H_5CH_2COO^-$ −hν	6
C(4a)-OH,N(5)-C_2H_5	4a,5-FAD_{red} (modified)					374	9.4	500		Chemical modif. of coenzyme	25
C(4a)-N(5)-propano	4a,5-FAD_{red} (modified)					382	6.5	518		Chemical modif. of coenzyme	25
L-Amino acid oxidase	FAD	460 383	11.3	—	—	450s 400s	2.5 4.5	520	3.2	$Na_2S_2O_4$, substrate	6
L-Lysine monooxygenase	FAD	462 385	11.2 11.2	—	—	410s 350	2.8 5.5	512	2.1	Lysine, $Na_2S_2O_4$	6
Melilolate hydroxylase	FAD	454 384	11.3 10.3	530	1.2	410 350	2.5 5.1	—	—	EDTA-hν	6
p-Hydroxybenzoate hydroxylase	FAD	450 374	11.3	525	1.2	420 350	2.1 5.1	—	—	EDTA-hν	6
C(4a)-OH,N(5)-C_2H_5	4a,5-FAD_{red} (modified)					370	9	500		Chemical modif. of coenzyme	25
C(4a)-OOHOH-OH,N(5)-H	4a,5-FAD_{red}					385	9			red. enzyme + O_2	28
Luciferase C(4a)-OOH,N(5)-H	4a,5-FMN_{red}					372	9	485–500		red. enzyme + O_2	29
Oxynitrilase	FAD	460 390	11.2 12.4	—	—	345	5.0	500	2.4	$Na_2S_2O_4$	6
Lipoic acid dehydrogenase	FAD	455 365 355	11.3 9.2 9.1	520	45	360	3.5	—	—	$Na_2S_2O_4$	6
Thioredoxin reductase	FAD	480s 457 380	9.3 11.3 11.7	520	15.5	425 320	2.4 9.0	—	—	$Na_2S_2O_4$, $NaBH_4$	6
Butyryl-CoA dehydrogenase	FAD	448 376	10.6 8.2	530	5.5	370s	3.4	520	0.8	$Na_2S_2O_4$	6
Flavodoxin *P. elsdenii*	FMN	447	10.6	—	—	450s 365	1.7 5.5	530	0.9	$Na_2S_2O_4$	6
		378	9.1			313	9.5				
Clostridium MP	FMN	447	10.6	—	—	450s 367	1.5 5.25	530	0.75	$Na_2S_2O_4$	6
		379	9.1			315	8.3				
Azotobacter vinelandii	FMN	452 372	10.6 9.6	—	—	d	d	—	—	$Na_2S_2O_4$	6
Old yellow enzyme	FMN	462 380	10.6 10.6	530	3.6	340	4.7	—	—	$Na_2S_2O_4$	6

[a] Modified and reprinted with permission from S. Ghisla, V. Massey, J. M. Lhoste, and S. G. Mayhew, *Biochemistry* 13, 589 (1974). Copyright by the American Chemical Society.

[b] Numbers refer to text footnotes.

of this chemical can obscure the spectrum of reduced flavins in the region where λ = <360 nm. Dithionite, however, should be used with caution, because variable amounts of sulfite are present as an impurity in commercial samples,[9] and sulfite is formed as an oxidation product of dithionite. Sulfite itself has been found to react with several flavoproteins, the species formed being covalent N(5) adducts, which have spectra similar to those of reduced enzyme flavins.[13] The usefulness of dithionite for the reduction of redox enzymes in general has been discussed recently in detail.[11]

Borohydride. This agent has the considerable advantage, as compared to dithionite, that it does not show any measurable absorption at λ = >300 nm, and can thus be used, if necessary, in large excess. In some cases, however, the isomeric 3,4-dihydroflavin might be obtained[14] instead of the 1,5-dihydro species, as desired. The reduction of free flavins by borohydride has been found to be catalyzed by light, while the reduction of flavoenzymes might be catalyzed by the presence of catalytic amounts of free Fl_{ox}. These reactions have been described earlier in this series.[14]

Hydrogen. The reduction of free flavins by hydrogen in the presence of catalysts leads to formation of 1,5-dihydroflavins. This method has the advantage that there are no by-products to interfere, with spectroscopic measurements. Most catalysts will, interfere, however, and must therefore be separated either by filtration or by centrifugation.[6] Charcoal, a carrier, for example, of metallic platinum or palladium catalysts, is known to efficiently adsorb flavins; thus caution is due when quantitative optical measurements are made. A good catalyst carrier for flavin reduction is silica. The catalytic method has been extensively used when large volumes of solutions of reduced flavins are needed, or when the reduction is carried out in organic solvents.[15]

Hydrogen has been used as reducing agent in homogeneous solution with hydrogenase as a catalyst.[9] Under particular conditions (strong acid), catalytic hydrogenation can lead to "overreduction," i.e., full hydrogenation of the flavin benzene moiety.[16]

Photoreduction. The photoreduction of free flavins and of flavoproteins in the presence of potential electron donors such as EDTA, amines, amino acids, and carboxylic acids has been known for several years,[17] and it also has been described earlier in this series.[18] In addition to the commonly used electron donors, such as those mentioned above, which might yield interfering products upon photooxidation, oxalate has been found to be an equally convenient reducing agent.[19] It has, moreover, the advantage of formally decaying to "clean products," i.e., CO_2 + 2 electrons.

Recently the photoreduction of flavoproteins has been reinvestigated by Massey *et al.* in detail.[20] It was found that this reaction can be catalyzed by the presence of very small amounts of free flavins, which are first reduced photochemically, and which subsequently transfer electrons to protein-bound Fl_{ox}.[20]

A similar method of indirect photoreduction which yields excellent results[21,22] has also been recently described. It takes advantage of the fact that catalytic amounts of deazaflavins form highly reactive 5-deazaflavin radicals upon illumination in the presence of electron donors (EDTA, oxalate):

$$\text{deazaFl}_{ox} \xrightarrow[\text{(EDTA)}]{h\nu} \underset{\text{(radical)}}{\text{deazaFl}} \longrightarrow 1e^- \text{ reduction of enzyme-bound } Fl_{ox}$$

This method is also particularly useful because it yields fully reduced flavoenzymes, whereas the direct photochemical reduction in the presence of EDTA does not go beyond the semiquinone state.

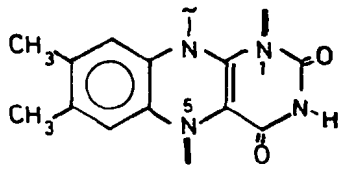
Reduction by Natural Substrate. Clearly this is the method of choice for the reduction of flavoproteins when full reduction does not require too large an excess of substrate, or when neither substrate nor products interferes with the envisaged measurements.

The Reduced Flavin Chromophore

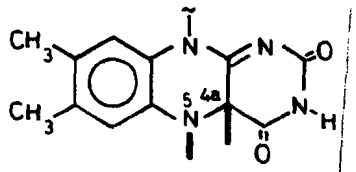
Two-electron reduction of Fl_{ox} can lead, formally, to several tautomeric forms of reduced flavin. To date only the 1,5-dihydro and the 4a,5-dihydro forms have been shown to be important in enzymic catalysis.

[13] F. Müller, V. Massey, and P. Hemmerich, this series, Vol. 18, Part B [ ], p. 474 and literature cited therein.
[14] F. Müller and V. Massey, this series, Vol. 18, Part [ ], p. 468 and literature cited therein.
[15] V. Favaudon, *Eur. J. Biochem.* **78**, 293 (1977).
[16] C. Heizmann, P. Hemmerich, R. Mengel, and W. Pfleiderer, *Helv. Chim. Acta* **56**, 1908 (1973).

[17] W. R. Frisell, C. W. Chung, and C. G. Mackenzie, *J. Biol. Chem.* **234**, 1297 (1959).
[18] C. Veeger, J. F. Koster, and D. B. McCormick, this series, Vol. 18, Part [ ], p. 509.
[19] S. Ghisla and V. Massey, *J. Biol. Chem.* **250**, 577 (1975).
[20] V. Massey, M. Stankovich, and P. Hemmerich, *Biochemistry* (in press).
[21] V. Massey and P. Hemmerich, *J. Biol. Chem.* **252**, 5612 (1979).
[22] V. Massey and P. Hemmerich, *Biochemistry* (in press).



1,5-Dihydroflavin



4a,5-Dihydroflavin

Structures derived from 1,6-, 1,8-, 1,3-, 10a-1 dihydroflavin species have been discussed at various times,[23] in particular in connection with the structure of possible adducts of reduced flavin and oxygen. C(4)-carbonyl reduced dihydroflavins,[13] and those which are derived from benzene ring hydrogenation,[16] do not play any biological role, and will thus be omitted from the discussion.

1,5-Dihydroflavins

The 1,5-Fl_{red} flavin chromophore is characterized by a rather structureless absorption in the visible and near-UV range, the shape of which is strongly influenced by temperature, the ionization state of the flavin, the properties of the solvent, and by the presence of substituents in particular at positions N(1) and N(5). The ionization at position N(1) ($pK \simeq 6.5$), causes a ≈50-nm hypsochromic shift of the longest wavelength maximum in aqueous media (Fig. 1). In ethanol and at ambient temperature, the effect of ionization is less pronounced (Fig. 2). While these species are nonfluorescent in solution and at ambient temperature, their incorporation in a glass matrix causes a white-bluish fluorescence to appear ($\lambda_{max} \simeq 500$ nm) which is the mirror image of the absorption spectrum recorded under the same conditions (Fig. 2).[6] Furthermore, under the conditions of Fig. 2, the absorption spectra now appear much better resolved and indicate that the 1,5-dihydroflavin chromophore has main $\pi \rightarrow \pi^*$ transitions centered at ≈400, ≈350, and ≈295 nm. Low-temperature spectra of N(1)- or N(5)-substituted dihydroflavins confirm this attribution.[6]

The peculiar variability of the absorption and fluorescence spectra of the 1,5-dihydroflavin chromophore probably originates in its "butterfly-shaped" structure.[6,24] This molecule can undergo a series of vibrations [e.g., N(5) and N(10) pyramidal nitrogen inversions, N(5)-N(10) ring inversion][24] leading to different conformational states. In their coplanar

[23] P. Hemmerich and A. Wessiak, *Flavins Flavoproeins, Proc. Int. Symp., 5th, 1975*, p. 9 (1976).

[24] L. Tauscher, S. Ghisla, and P. Hemmerich, *Helv. Chim. Acta* **56**, 630 (1973).

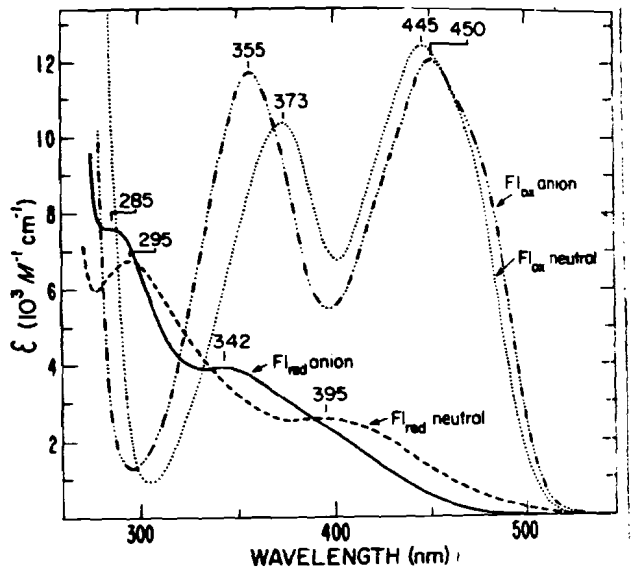


FIG. 1. Absorption spectra of flavin mononucleotide (FMN) in aqueous solution in th oxidized and reduced state and in the neutral and anionic state. FMN_{ox} in 0.033 *N* NaO (—···—); FMN_{ox} at pH 5 in 0.09 *M* citrate buffer (····); FMN_{red} at pH 8.5 in 0.1 pyrophosphate buffer (———); FMN_{red} at pH 5 in 0.09 *M* citrate buffer (---). The redu tions were achieved by illumination in the presence of EDTA. Adapted with permissio from S. Ghisla, V. Massey, J. M. Lhoste, and S. G. Mayhew, *Biochemistry* **13**, 589 (1974 Copyright by the American Chemical Society.

state, 1,5-dihydroflavins are "antiaromatic" (8 π electrons in the pyrazin ring), while in a "bent conformation" the tricyclic resonance of th isoalloxazine system might be restricted. Thus, depending on the degre of "bending" around the N(5)-N(10) axis, the allowedness of sever electronic transitions might be greatly influenced, and result in absorptic spectra of very different shapes.

4a,5-Dihydroflavins

The near-UV absorption spectrum of the 4a,5-dihydroflavin chror ophore is similar, in its appearance, to that of its 1,5-dihydro isomer, b its shape is even more dependent on temperature and solvent (Fig. 3). particular, substituents which have an effect on the internal mobility the molecule were found to cause major spectral changes on the chror ophore free in solution,[6] and also when bound to various apoflavopr

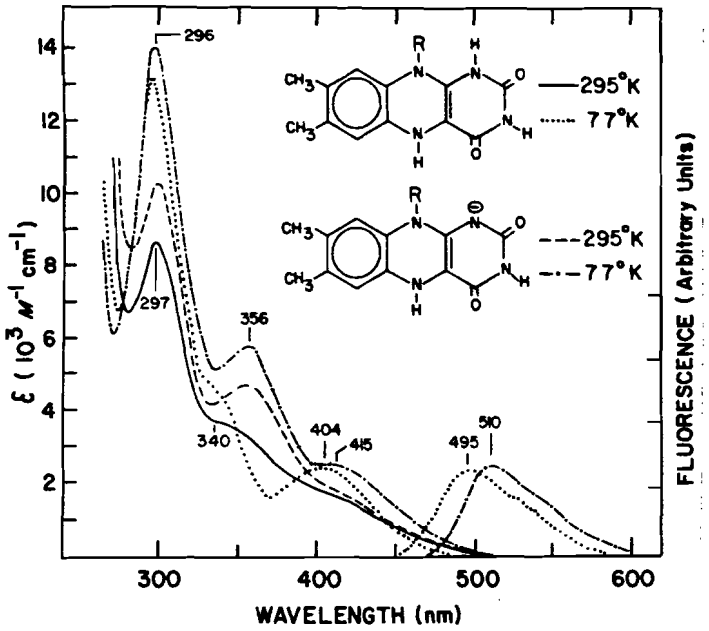


FIG. 2. Absorption and fluorescence spectra of reduced tetraacetylriboflavin [R = $CH_2(CHOAc)_3CH_2OAc$] in ethanol at room (295°K) and liquid nitrogen (77°K) temperature. The anion was obtained in the presence of 5% triethylamine. The extinction coefficients refer to the room temperature spectra. They must be corrected for solvent contraction at 77°K. The reduction was carried out with H_2-Pt, and the samples were sealed under vacuum in 4-mm OD quartz tubes. Adapted with permission from S. Ghisla, V. Massey, J. M. Lhoste, and S. G. Mayhew, *Biochemistry* 13, 589 (1974). Copyright by the American Chemical Society.

teins.[25] At ambient temperature, 4a,5-dihydroflavins show a weak fluorescence only when an appropriate substituent (e.g., 4a,5-propanobridge) decreases their internal mobility.[6] However, at low temperature in rigid ethanol glasses,[6] or when bound to apoflavoproteins,[25] 4a,5-dihydroflavins exhibit a rather strong fluorescence emission maximal in the range 480–530 nm (see the table). As in the case of 1,5-dihydroflavins, the 4a,5-dihydro chromophore exhibits at least three transitions in the near-UV range (≈285, ≈310, and ≈380 nm), which are reasonably attributed to $\pi \rightarrow \pi^*$ excitations.[6]

[25] S. Ghisla, B. Entsch, V. Massey, and M. Husein, *Eur. J. Biochem.* 76, 139 (1977).

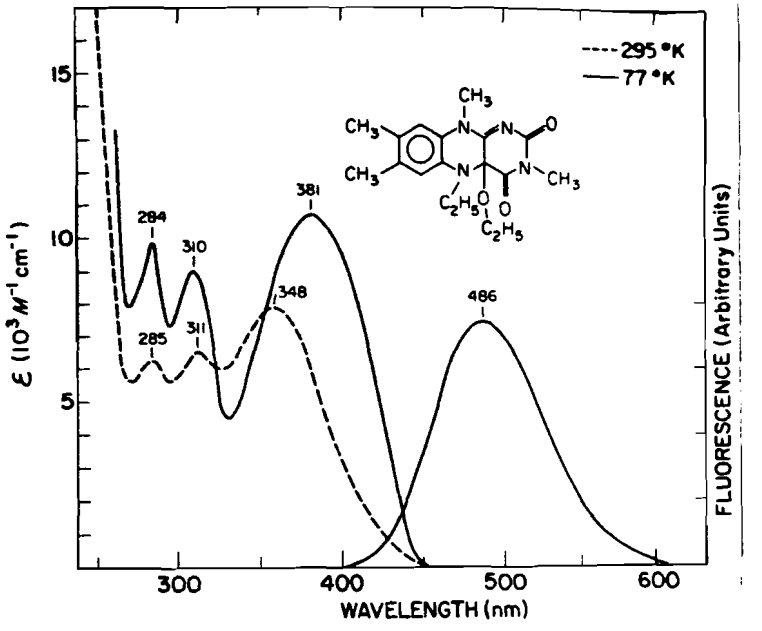


FIG. 3. Absorption and fluorescence spectra of 4a,5-disubstituted FL_{red} in ethanol at room (295°K) and liquid nitrogen temperature (77°K). Adapted with permission from S. Ghisla, V. Massey, J. M. Lhoste, and S. G. Mayhew, *Biochemistry* 13, 589 (1974). Copyright by the American Chemical Society.

Absorption and Fluorescence Properties of Reduced Flavoproteins

The spectral variability observed with free reduced flavin compounds[6] (Figs 1, 2, and 3) is even more pronounced among flavoproteins. In fact it is rare to find two reduced flavoenzymes, even within the same enzyme class, with closely similar absorption spectra. Figures 4 and 5, depicting the spectra of reduced lactate oxidase and flavodoxin from *P. elsdenii*, illustrate this point by showing two extreme cases. Nevertheless, by comparison with appropriate model compounds, and by choice of the correct conditions (temperature, solvent, ionization state), the spectra of most reduced flavoenzymes could be simulated.[6] Thus, the spectra of reduced 1,5-dihydro flavoenzymes might yield information about the ionization state of the reduced coenzyme and the protein environment at the active center. For example, the spectrum of reduced lactate oxidase (Fig. 4) is reminiscent of the spectra of 1- and 5-substituted 1,5-dihydroflavins,[6] i.e., of model compounds which exhibit a relatively large dihedral angle

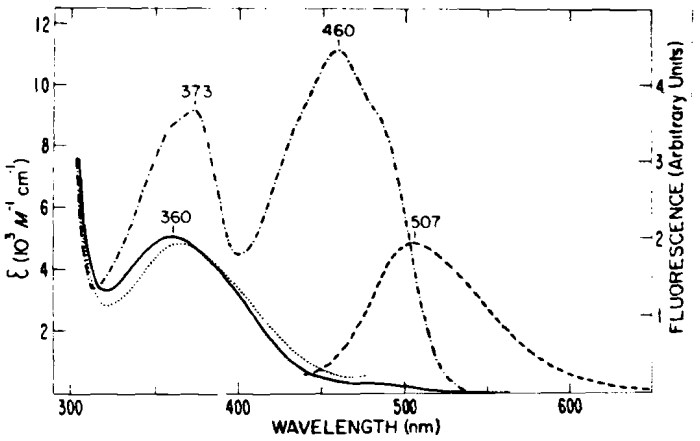


FIG. 4. Absorption, fluorescence emission, and fluorescence excitation spectra of lactate oxidase, 3×10^{-5} *M* in 0.01 *M* imidazole-HCl buffer (pH 7.0) at 25°. Absorption spectrum of the oxidized enzyme (—·—·—); absorption of the enzyme reduced with L-lactate, after the decay of the initially formed, nonfluorescent complex (———); fluorescence emission $\lambda_{excitation}$ 360 nm) (----); fluorescence excitation spectrum ($\lambda_{emission}$ 507 nm) of the reduced enzyme (····). The oxidized enzyme does not show detectable fluorescence. Adapted with permission from S. Ghisla, V. Massey, J. M. Lhoste, and S. G. Mayhew, *Biochemistry* **13**, 589 (1974). Copyright by the American Chemical Society.

(bending along the N(5)-N(10) axis to form a butterfly wing structure) between the pyrimidine and the benzenoid ring planes.[26] The spectrum of reduced flavodoxins (Fig. 5 and the table) resembles those of reduced flavins having a more planar configuration.[27]

As compared to 1,5-dihydro reduced flavoenzymes, considerably less information exists on protein-bound derivatives of 4a,5-dihydro reduced flavins or their derivatives. Among the biologically relevant cases, to date only the catalytic oxygen adducts involved in aromatic hydroxylations,[28] and in bacterial luminescence,[29] have been found to have a 4a,5-dihydroflavin structure. Several modified coenzymes having the same 4a,5-dihydroflavin chromophores have since been bound to apoflavoen-

[26] P. Kierkegaard, R. Norrestam, P. Werner, I. Csöregh, M. von Glehn, R. Karlsson, M. Leijonmark, O. Rönnquist, B. Stensland, O. Tillberg, and L. Torbjörnsson, *in* "Flavins and Flavoproteins" (H. Kamin, ed.), p. 1. Univ. Park Press, Baltimore, Maryland, 1971.

[27] K. H. Dudley, A. Ehrenberg, P. Hemmerich, and F. Müller, *Helv. Chim. Acta* **47**, 1354 (1964).

[28] B. Entsch, D. Ballou, and V. Massey *J. Biol. Chem.* **251**, 2550 (1976).

[29] W. Hastings, C. Balny, and P. Douzou, *Flavins Flavoproteins, Proc. Int. Symp., 5th, 1975* p. 53 (1976).

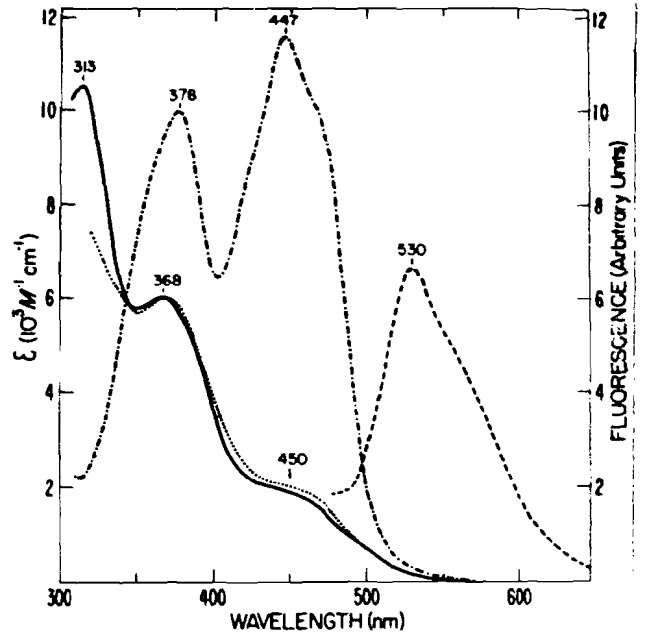


FIG. 5. Absorption, fluorescence emission, and fluorescence excitation spectra of flavodoxin from *P. elsdenii*, 2×10^{-5} *M*, in 0.1 *M* pyrophosphate buffer (pH 8.5) at 25°. Absorption of the oxidized enzyme (—·—·—); absorption spectrum of the enzyme reduced with dithionite in the apparatus described by Fourst *et al.* (———) [G. P. Foust, B. D. Burleigh, S. G. Mayhew, C. H. Williams, and V. Massey, *Anal. Biochem.* **27**, 530 (1969)]; fluorescence emission ($\lambda_{excitation}$ 368 nm) (----); fluorescence excitation spectrum ($\lambda_{emission}$ 530 nm) of the reduced enzyme (····). The oxidized enzyme is devoid of fluorescence. Adapted with permission from S. Ghisla, V. Massey, J. M. Lhoste, and S. G. Mayhew, *Biochemistry* **13**, 589 (1974). Copyright by the American Chemical Society.

zymes in order to study their spectral properties (see the table).[25,30] The effects observed upon complexation with the protein are even more pronounced than those observed within 1,5-dihydroflavoproteins. A typical case is illustrated in Fig. 6, where upon binding to the apoenzyme of D-amino acid oxidase the absorption of 4a-hydroxy-5-ethyl-FAD (compare to Fig. 3) is modified to a single-banded spectrum in the near-UV range (Fig. 6), and the extinction coefficient is increased by ≈30%. A qualitatively similar effect is observed with the 4a,5-dihydroflavin model of Fig. 3 upon freezing into a glassy matrix.

[30] S. Ghisla, H. Ogata, V. Massey, A. Schonbrunn, R. H. Abeles, and C. T. Walsh, *Biochemistry* **15**, 1791 (1976).

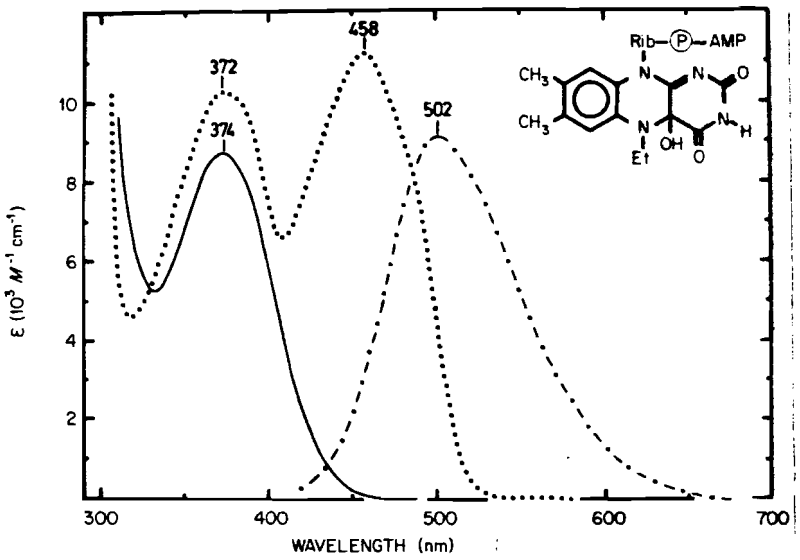


FIG. 6. Absorption and fluorescence emission spectrum of 4a-hydroxy-5-ethyl-FAD bound to D-amino acid apooxidase. The complex was prepared by incubation for 10 min at 0° of 0.5 ml 10^{-4} *M* D-amino acid apooxidase with 10-fold excess of the modified FAD in 0.05 *M* phosphate buffer, pH 7.5.[3] The protein was separated from excess coenzyme by chromatography over a short Sephadex G-25 equilibrated with the same buffer, and at 0–4°. Curve (———), absorption spectrum, and curve (—·—·), fluorescence emission spectrum of the complex, 2×10^{-5} *M*. The excitation wavelength was 375 nm. The excitation spectrum, recorded with $\lambda_{emission} = 500$ nm, closely follows the absorption spectrum. Curve (····) shows the spectrum of the oxidized enzyme under the same conditions for comparison.

Note that spectral effects such as those shown in Fig. 6 are most probably not due to chemical modification of the 4a,5-dihydroflavin chromophore upon binding to the apoprotein, as has been observed in certain cases.[30] In fact the 4a-hydroxy-5-ethyl-4a,5-dihydro chromophore could be recovered intact after such experiments upon denaturation of the protein.[25] We observed recently that, in contrast to common beliefs, reduced flavoproteins can be fluorescent.[6] Among the examples listed on the table, roughly one-half were found to exhibit measurable fluorescence emission in the range 460–530 nm. This was taken as an indication[6] that the reduced flavin coenzyme might be placed in a rigid environment at the enzyme-active center. Similarly, at least some of the inversion processes occurring in solution[24] might be slowed down to such an extent that they do not give a major contribution to radiationless energy transfer. Supporting this hypothesis is the fact that in several cases introduction of substituents, which will enhance rigidity of the reduced flavin, has

been found to increase the fluorescence yield (see the table).[6,25,31] No correlation between occurrence of fluorescence and function of the flavoenzyme has been found. On the other hand, a general trend can be seen where those enzymes that are fluorescent in the oxidized state are not in the reduced state, and vice versa.

Finally, we wish to emphasize the potential usefulness of fluorescence studies for the investigation of flavoenzyme reaction mechanisms, where transient intermediates having the reduced flavin chromophore might be accessible to characterization. In fact, recently covalent intermediates occurring during the reaction of lactate oxidase with glycolate could be demonstrated by taking advantage of their strong fluorescence emission at 465 nm.[31,32]

Acknowledgment

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[31] V. Massey and S. Ghisla, *Proc. 10th FEBS Meet.* p. 145 (1975).
[32] S. Ghisla and V. Massey, *in* "Mechanisms of Redox Enzymes" (R. Ondarza and T. P. Singer, eds.). Am. Elsevier, New York (in press).